ANTIBIOTIC PRODUCTON

REFERENCE TO PROVISIONAL APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/242,561 filed on October 23, 2000, the entire disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to methods and materials for controlling antibiotic production in species of *Streptomyces*, especially increasing antibiotic production in *Streptomyces coelicolor* and *S. lividans*.

INTRODUCTION

In addition to undergoing a complex process of morphological differentiation, streptomycetes are renowned for their ability to produce a vast array of secondary metabolites, many of which possess antibiotic or other pharmacologically useful activities. Most of these secondary metabolites are the products of complex biosynthetic pathways that are activated in a growth phase-dependent manner. While the production of antibiotics in liquid culture is generally limited to stationary phase, in surface-grown cultures it usually coincides with the onset of morphological differentiation (Chater and Bibb, 1997).

In several streptomycetes, γ-butyrolactones (GBLs) have been shown to play important, if not crucial, roles in determining the onset of antibiotic production and morphological differentiation (Horinouchi and Beppu,

1994; Yamada, 1999). The most characterised γ-butyrolactone is A-factor (2-isocaryloyl-3R-hydroxymethyl-γ-butyrolactone), which is required for both streptomycin production and sporulation in *Streptomyces griseus* (Mori, 1983; Horinouchi and Beppu, 1994). Other well-studied γ-butyrolactones include virginiae butanolides (VB), which appears to control virginiamycin production in *Streptomyces virginiae* (Yamada *et al.*, 1987; Kondo *et al.*, 1989), and IM-2, which elicits the production of showdomycin and minimycin in *Streptomyces lavendulae* FRI-5 (Sato *et al.*, 1989).

Although the details of A-factor synthesis have not been elucidated, a putative A-factor biosynthetic gene, *afsA*, was cloned from *S. griseus* and sequenced. Its predicted translation product does not resemble any protein of known function (Horinouchi *et al.*, 1989). *afsA* mutants of *S. griseus* are deficient in A-factor synthesis, and hence in streptomycin production and sporulation. Moreover, cloning of *afsA* in multiple copies leads to precocious streptomycin production in *S. griseus*, and to the production of a compound with A-factor activity in other streptomycetes that normally do not make it (Horinouchi *et al*, 1985). Culture supernatants of an *Escherichia coli* strain over-expressing *afsA* restored streptomycin production and sporulation in an A-factor-deficient mutant of *S. griseus* (Ando *et al.*, 1997).

A-factor is detected in culture supernatants of *S. griseus* just before the onset of streptomycin production. It diffuses freely across the cytoplasmic membrane, and binds with high affinity to a cytoplasmic A-factor-binding protein, ArpA (Onaka *et al*, 1995). In the absence of A-factor, ArpA acts as a negative regulator of both streptomycin production and sporulation by

repressing transcription of the pleiotropic regulatory gene *adpA* (Ohnishi *et al.*, 1999). Homologues of *afsA* and/or *arpA* have been isolated from several streptomycetes, including *S. virginiae* (Okamoto *et al.*, 1995; Kinoshita *et al.*, 1997), *S. lavendulae* (Waki *et al.*, 1997), *S. coelicolor* (Onaka *et al.*, 1998) and S. *fradiae* (Fouces *et al.*, 1999; Bate *et al.*, 1999).

S. coelicolor is the most genetically characterised streptomycete. It produces at least four chemically distinct antibiotics. Two of these, actinorhodin (Act) and undecylprodigiosin (Red), are pigmented. The stationary phase production of Act and Red results from transcriptional activation of the pathway-specific activator genes *act*II-ORF4 and *redD*, respectively (Gramajo *et al.*, 1993; Takano *et al.*, 1992). Moreover, production of Act and Red in exponential phase appears to be prevented only by the absence of a threshold concentration of the pathway specific activator proteins.

Recently, four extracellular compounds were identified in culture supernatants of *Streptomyces coelicolor* A3(2) that elicited the precocious production of the antibiotics actinorhodin (Act) and undecylprodigiosin (Red) when added to the producing strain; none of the compounds induced morphological differentiation. One of these stimulatory factors, SCB1, was purified to homogeneity and shown by structural elucidation to be a γ-butyrolactone (Takano *et al.*, 2000).

SUMMARY OF THE INVENTION AND DETAILED DESCRIPTION

The present inventors have identified genes of *S. coelicolor* which are involved in the regulation of Act

and Red production. One gene, *scbA*, is a homologue of *afsA* (A-factor synthetase of *S. griseus*), and the other, *scbR*, encodes a γ-butyrolactone binding protein. By analogy with the *S. griseus* system, ScbR was expected to be a repressor of the pathway-specific activator genes *act*II-ORF4 and *redD*. Release of such repression upon binding of ScbR by the GBL SCB1 at high cell densities would lead to antibiotic expression.

However, it was found that ScbR binds to the transcription start sites of *scbA* and *scbR*, and is released by addition of SCB1 from *S. coelicolor*. An in-frame deletion mutant of *scbA* (a gene involved in GBL synthesis) shows overproduction of Act and Red (when lack of antibiotic production might have been expected) and an in-frame deletion mutant of *scbR* shows delay in Red production and earlier Act production (*scbR* - see Fig. 5A). These phenotypes therefore differ from what might be expected by analogy to the *S. griseus* A-factor system.

Moreover, the inventors have found that *S. lividans* strains carrying the same in-frame deletion mutant of *scbA*, in place of the wild-type *scbA* gene usually present in *S. lividans*, also overproduce Act and Red.

The inventors propose, therefore, that mutations to homolgues of *scbA* and *scbR* in other *Streptomyces* species may have similar effects.

Accordingly, in a first aspect, the present invention provides a method of modifying an antibiotic-producing strain of a *Streptomyces* species to increase antibiotic production in said strain, the method comprising functionally deleting in said strain a gene which is the *scbA* gene of *Streptomyces coelicolor* or a homologue

thereof.

In a second aspect, the present invention provides a method of modifying an antibiotic-producing strain of a *Streptomyces* species to alter the timing of antibiotic production in said strain, the method comprising functionally deleting in said strain a gene which is the *scbR* gene of *Streptomyces coelicolor* or a homologue thereof.

While it is appreciated that these effects will not be found in relation at least to streptomycin production in *S. griseus*, and perhaps in some other *Streptomyces* species, it is thought that the effects may not be confined to the exemplified species of *S. coelicolor* and *S. lividans*. It will be possible for the skilled person to repeat the experimental disclosure presented herein on other *Streptomyces* species, thereby to identify other species in which similar effects occur. In particular, it will be possible to identify in other species of *Streptomyces* genes which are homologues of *scbR* and *scbA* in an analogous way to the identification herein of *scbR* and *scbA*. Following identification of the genes, it will be possible to create strains in which these genes are functionally deleted, and to compare the extent and/or timing of antibiotic production in those modified strains with the extent and/or timing of production in the parent strain. Those modified strains in which similar effects are found to those presented herein are regarded also to be part of the invention.

Accordingly, in a third aspect, the present invention provides a modified strain of a *Streptomyces* species, the modified strain having a functional deletion of a gene which is the *scbA* gene of *S. coelicolor* or a homologue

thereof, whereby production of at least one antibiotic in said modified strain is increased compared to a wild-type strain of said *Streptomyces* species.

Similarly, in a fourth aspect, the present invention provides a modified strain of a *Streptomyces* species, the modified strain having a functional deletion of a gene which is the *scbR* gene of *S. coelicolor* or a homologue thereof, whereby the timing of production of at least one antibiotic in said modified strain is altered compared to a wild-type strain of said *Streptomyces* species.

In a fifth aspect, the present invention provides a method of producing an antibiotic, the method comprising providing a modified *Streptomyces* strain of any preceding aspect, and culturing said strain under conditions suitable for production of antibiotic.

The method may also comprise the additional step of purifying the antibiotic from the culture medium. It may also comprise the further step of formulating the antibiotic as a pharmaceutical.

The *scbR* and *scbA* genes are believed to be new, as is a further gene, designated *scbB*, which is downstream of *scbR* and which shows homology to the C-5 ketoreductase gene of *S. avermitilis*. *scbB* is predicted to modify the C-6 of SCB1 from keto to hydroxyl, and may therefore be important in providing specificity of SCB1 as the cognate GBL of scbR.

In a sixth aspect, the present invention provides a nucleic acid comprising a nucleotide sequence having at least about 80% identity with a nucleic acid sequence selected from the group consisting of (1) nucleotides

3032 to 3679, (2) nucleotides 2914 to 1970, and (3) nucleotides 4529 to 3795, reading 5' to 3', of the nucleic acid deposited as EMBL AJ007731, which may alternatively be defined as (1) nucleotides 2261-2908, (2) nucleotides 2142-1199 and (3) nucleotides 3758-3024, respectively of Fig. 14.

As will be evident from Fig. 1, both strands of DNA in this region encode polypeptides. Nucleotide numbering is given in relation to the strand which runs from 5' to 3' from right to left in Fig. 1. The sequence of part of this strand is given in Fig. 14. However, the coding sequences of *scbA* and *scbB* are on the complementary strand. References to the nucleotide sequences in EMBL AJ007731 and Fig. 14 which relate to these genes (i.e. where the nucleotide numbering is shown as [higher number]-[lower number]) should therefore be interpreted as references to the strand complementary to that shown.

Preferably the nucleic acid sequence identity is at least 85%, 90%, 95%, 98% or 99% or is 100%.

In a seventh aspect, the present invention provides a nucleic acid comprising a nucleotide sequence which encodes a polypeptide having at least about 70% amino acid sequence identity with an amino acid sequence selected from the group consisting of (1) the amino acid sequence of ScbR, as shown in Fig. 9, (2) the amino acid sequence of ScbA, as shown in Fig. 10, and (3) the amino acid sequence of ScbB, as shown in Fig. 11.

In further aspects, the present invention provides: polypeptides encoded by the nucleic acid molecules of the sixth and seventh aspects; vectors including the nucleic acids of those aspects, optionally in operative

association with control sequences, e.g. promoter and/or enhancer sequences; host cells transfected with said vectors; and methods of producing said polypeptides, comprising culturing said host cells under conditions suitable for polypeptide production and extracting said polypeptides from the culture medium.

In a still further aspect, the present invention provides a method for identifying *Streptomyces* species in which antibiotic production is increased by functionally deleting the *scbA* gene of *S. coelicolor* or a homologue thereof, the method comprising functionally deleting in an antibiotic-producing strain of a *Streptomyces* species the *scbA* gene of *S. coelicolor* or a homologue thereof, culturing said strain under conditions suitable for the production of antibiotic, and determining whether antibiotic production in said strain is increased.

Similarly, the invention also provides a method for identifying *Streptomyces* species in which the timing of antibiotic production is altered by functionally deleting the *scbR* gene of *S. coelicolor* or a homologue thereof, the method comprising functionally deleting in an antibiotic-producing strain of a *Streptomyces* species the *scbR* gene of *S. coelicolor* or a homologue thereof, culturing said strain under conditions suitable for the production of antibiotic, and determining whether the timing of antibiotic production in said strain is altered.

In a further aspect, the invention provides a method for producing an antibiotic, the method comprising, following identification of a *Streptomyces* species according to the preceding aspect, providing a strain of said species having a functional deletion of said *scbA* or *scbR* gene of

S. coelicolor or homologue thereof, and culturing said strain under conditions suitable for antibiotic production.

As before, the method may further comprise the step of purifying the antibiotic from the culture medium. It may also comprise the step of formulating the antibiotic as a pharmaceutical.

As used herein, the term "functional deletion" of a gene may mean any alteration of the nucleic acid in a cell or cells of the strain containing the functional deletion, which alteration has the effect of preventing normal expression of that gene. For example, the gene may comprise a deletion in the coding sequence, leading to a shortened transcript which is translated into a protein lacking the normal function of the expression product of the gene; or the transcriptional and/or translational regulatory sites (e.g. promoter and/or enhancer sequences) may be altered to prevent normal transcription and/or translation of the gene; or the coding sequence may contain an insertion or mutation (e.g. to introduce or produce a stop codon or to cause a shift in reading frame), leading to a non-functional expression product. Alterations of the coding sequence may be in frame or may cause a shift in reading frame. As a further alternative, the cell(s) may be modified to produce antisense mRNA, which prevents correct translation, preventing gene expression even if the gene itself is unmodified.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is

complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Rothstein et al, 1987; Smith *et al*,(1988) *Nature* 334, 724-726; Zhang *et al*,(1992) *The Plant Cell* 4, 1575-1588, English *et al.*, (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

An alternative is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression. See, for example, van der Krol *et al.*, (1990) *The Plant Cell* 2, 291-299; Napoli *et al.*, (1990) *The Plant Cell* 2, 279-289; Zhang *et al.*, (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene. Total complementarity or similarity of sequence is not essential. The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is

about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the target gene.

Preferred *Streptomyces* species for the practice of the invention are species which possess adjacent and divergent *scbA* and *scbR* genes of *S. coelicolor* or adjacent and divergent homologues thereof, since it is thought that this arrangement of genes may correlate with the effects on amount and timing of antibiotic production seen in *S. coelicolor* and *S. lividans*.

The closely related species *S. coelicolor*, *S. violaceoruber*, *S. lividans* and *S. parvulus* are particularly preferred. Strains of such species (i.e. wild-type strains) are commonly available, e.g. from the ATCC, for example under ATCC deposit numbers 12434 for *S. parvulus* and 19832 for *S. violaceoruber*. *S. coelicolor* A3(2) and *S. lividans* 66 are particularly preferred wild-type strains and are available from the John Innes Culture Collection (Norwich, UK) under JICC deposit numbers 1147 and 1326, respectively. However, the invention is not limited to such particular strains.

The present invention may exclude the modification of *barX* and/or *farX*, the *afsA* homologues in *S. virginiae* and *S. fradiae*, respectively.

A gene of a *Streptomyces* species or strain, which gene is a "homologue" of or is "homologous" to the *scbA* gene of *S. coelicolor*, may be the gene which shows greatest deduced amino acid sequence identity to *scbA* of all genes of said species or strain; alternatively or additionally, it may be a gene which is capable of specific hybridisation with the amplification product obtained using the primers oligo1 (5'-GACCACGT(CG)CC(CG)GGCATG)

and oligo2 (5'-GTCCTG(CG)TGGCC(CG)GT(CG)AC(CG)CG(CG)AC) to amplify total DNA of said species or strain (bracketed nucleotides indicate positions of degeneracy); alternatively or additionally, it may be a gene encoding a polypeptide having at least about 35% sequence identity with the deduced amino acid sequence of *scbA* as shown in Fig. 10, preferably at least about 40% (which is the homology found between *scbA* and other homologues of the *afsA* gene of *S. griseus*) more preferably about 50%, 60%, 65% (which is the homology found between *scbA* and *afsA* of *S. griseus*), 70%, 80%, 90%, or 95%.

A gene of a *Streptomyces* species or strain, which gene is a "homologue" of or is "homologous" to the *scbR* gene of *S. coelicolor*, may be the gene which shows greatest deduced amino acid sequence identity to *scbR* of all genes of said species or strain; alternatively or additionally, it may be a gene which is adjacent to and divergent from a gene which is capable of specific hybridisation with the amplification product obtained using the primers oligo1 (5'-GACCACGT(CG)CC(CG)GGCATG) and oligo2 (5'-GTCCTG(CG)TGGCC(CG)GT(CG)AC(CG)CG(CG)AC) to amplify total DNA of said species or strain (bracketed nucleotides indicate positions of degeneracy); alternatively or additionally, it may be a gene encoding a polypeptide having at least about 35% sequence identity with the deduced amino acid sequence of *scbR* as shown in Fig. 9, preferably at least about 40%, more preferably about 45% (which is the homology found between *scbR* and *arpA* of *S. griseus*), 50%, 55% (which is the homology found between *scbR* and the *FarA* gene of *S. lavendulae*) 60%, 65%, 70%, 80%, 90%, or 95%.

"Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues in a candidate

sequence that are identical with the amino acid residues in the sequence with which it is being compared, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. The % identity values used herein are generated by WU-BLAST-2 which was obtained from Altschul et al. (1996); http://blast.wustl/edu/blast/README.html. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSPS and HSPS2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region, multiplied by 100. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-BLAST-2 to maximize the alignment score are ignored).

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the sequence under comparison. The identity values used herein were generated by the BLASTN module of WU BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

Methods of genetically manipulating *Streptomyces*,

culturing *Streptomyces* under conditions suitable for antibiotic production and purifying antibiotics from *Streptomycete* cell culture medium are well known to the skilled person, e.g. from Hopwood et al. (1985) and Kieser et al (2000).

Similarly, methods of formulating antibiotics as pharmaceuticals are well known in the art. Such pharmaceutical formulations may comprise, in addition to the antibiotic, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, transdermal, transmucosal, intramuscular, intraperitoneal routes.

Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the antiobiotic will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Formulations suitable for transmucosal administration include liquids, solutions, suspensions, emulsions, suppositories, pessaries, gels, pastes, ointments, creams, lotions, oils, as well as patches, adhesive plasters, depots, and reservoirs.

Formulations suitable for transdermal administration include gels, pastes, ointments, creams, lotions, and oils, as well as patches, adhesive plasters, bandages, dressings, depots, and reservoirs.

Ointments are typically prepared from the active compound and a paraffinic or a water-miscible ointment base.

Creams are typically prepared from the antibiotic and an oil-in-water cream base. The aqueous phase of the cream base may include at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the active compound through the skin or

other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

Formulations may suitably be provided as a patch, adhesive plaster, bandage, dressing, or the like which is impregnated with one or more active compounds and optionally one or more other pharmaceutically acceptable ingredients, including, for example, penetration, permeation, and absorption enhancers.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences (*supra*).

A pharmaceutical formulation may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 Restriction map of 7.5kb DNA fragment isolated from *S. coelicolor* which includes *scbA* and *scbR*. The positions of *scbA*, *scbR*, *orfX* (also referred to herein as *scbB*) are indicated by shaded boxes and the other ORFs with no apparent homology to other known streptomyces antibiotic regulatory genes (as assessed by the BLAST program) are indicated by open boxes. The ORFs are deduced from the FRAME program (Bibb *et al.*, 1983). pIJ6111 and pIJ6114 were used for sequencing analysis of the 7.5kb fragment. Restriction maps of the in-frame deletion mutant constructs are also shown. Dotted lines indicate the in-frame deletion of *scbA* for pIJ6120 or *scbR* for pIJ6124. The *Pst*I site in pIJ6124 was generated by using a designed primer for PCR to allow ligation with the corresponding *Pst*I site. The *BamH*I site in pIJ6140 was end filled and ligated with *Pvu*II. Single arrows (PrimersR) and double arrows (PrimersA) denote the primers used to determine the *scbR* and *scbA* mutation, respectively, after the second crossover event. pIJ 6135 and pIJ6143 (insert cloned into pSET152) were used to complement the *scbR* and *scbA* mutant, respectively. pIJ6120 (insert in pIJ2925) was used to express ScbR for gel retardation and Dnase I footprinting experiments.

Fig. 2a S1 nuclease mapping of the transcriptional start site of *scbA* and *scbR*. Asterisks indicate the probable start points of the transcription; the sequences given are those of the template strand. Lanes T, G, C, and A are sequence ladders derived from the same primers as the probe generated by PCR and using the Taqtrack kit along with the these

primers.

Fig. 2b S1 nuclease mapping of *scbA*, *scbR*, and the major sigma factor *hrdB*, using RNA isolated from a liquid time course of *S. coelicolor* M145 at the time (hours) indicated. The EXP, TRANSITION and STAT indicated the exponential, transition and stationary phases of growth, respectively, and the shaded box labelled RED denote the presence of undecylprodigiosin in the mycelium; SM, end-labelled *Hpa*II-digested pBR322 size marker.

Fig. 3a Gel retardation experiment shown with crude extract of *E. coli* JM101 harboring *scbR*. Various crude extracts or unlabelled DNA fragments that were used in the experiment are indicated as +.

Fig. 3b Gel retardation experiments using *E. coli* JM101 crude extract harboring *scbR* and various γ-butyrolactones are indicated. CD denotes Circular Dichroism positive (+) or negative (-).

Fig. 4a Dnase I footprinting experiment of ScbR. Protection of the *scbA* and *scbR* promoter region by ScbR from cleavage by Dnase I is shown by vertical lines. No.1 denotes ScbR binding site No.1 and No.2 binding site No.2. Both DNA strands were tested for protection by Dnase I by using two different ^{32}P labelled oligonucleotides. The A and G sequence ladder were used as size standards. Asterisk indicates the oligonucleotide which has been labelled. The presence or absence of crude extracts from *E. coli* JM101 harboring *scbR* is indicated by + or -. The numbers underneath the symbol > denote the concentration gradient of the crude extract added to

the reaction.

Fig. 4b ScbR binding sites No.1 and No.2 from Dnase I footprinting experiments. The protected sequences are indicated by lines and the numbering is with respect to the transcriptional start site of *scbA* for binding site No.1 and *scbR* for binding site No.2. The arrows and *pscbA*, *pscbR* indicate the transcriptional start site and direction of *scbA* and *scbR*, respectively. [ScbA] and [ScbR] indicate the coding sequence for *scbA* and *scbR*, respectively.

Fig. 5a Effect of deletion of *scbA* or *scbR* on antibiotic production in solid grown *S. coelicolor* M145. Confluent lawn of M145, M751 and M752 were grown on SMMS at 30°C for 20h (top plates) or 40h (bottom plates).

Fig. 5b Effect of deletion of *scbA* or *scbR* on response to SCB1, a γ-butyrolactone. Bioassay using spore suspension of M145(top), M751(left bottom) and M752(right bottom) as indicator strain and spotted with 1 µg of SCB1. The plates were incubated at 30°C for 20h (left panel) or 40h (right panel).

Fig. 6 Effect of deletion of *scbA* or *scbR* on the production of γ-butyrolactones with antibiotic stimulatory activity. Bioassay of ethyl acetate extracts from SMMS solid cultures of M145 (*pset*152) (top), M751 (*pset*152) (left side) and M751 complemented with *scbA* (right side), M752 (*pset*152) (right side) and M752 complemented with *scbR* (left side). In each case the indicator lawn is M145 and grown on SMMS at 30°C for 30h.

Fig. 7a S1 nuclease mapping of *scbA*, *scbR*, and the major sigma factor *hrdB*, using RNA isolated from a liquid time course of *S. coelicolor* M145, M751 and M752 at the numbers indicated. The E, TRAN and S indicates the exponential, transition and stationary phases of growth, respectively, and the shaded box labelled RED and A denote the presence of undecylprodigiosin and actinorhodin in the mycelium. The numbers in the box denotes the measurement of antibiotic production, ACT for actinorhodin, and RED for undecylprodigiosin, respectively. Numbers in bold refer to the time points when the antibiotics were measured, which corresponds to the time of RNA isolation.

Fig. 7b Bioassay of supernatants isolated at the time of RNA isolation from M145. The numbers denotes the different time points indicated in Figure 7a.

Fig. 8 S1 nuclease mapping of *scbA*, *scbR*, and the major sigma factor *hrdB*, using RNA isolated from M571 grown on liquid media SMM, with 0 or 31.25ng final concentration addition of SCB1.

Fig. 9 Deduced amino acid sequence of ScbR.

Fig. 10 Deduced amino acid sequence of ScbA.

Fig. 11 Deduced amino acid sequence of ScbB.

Fig. 12 Production of Act by *S. lividans* strains carrying pIJ68.

Fig. 13 Production of Red by *S. lividans* strains carrying pIJ6014.

Fig. 14 Nucleic acid sequence of region containing *scbA*, *scbR* and *scbB*. M751 (Δ*scbA*) is deleted from nt position 1320 to 2021; M752 (Δ*scbR*) is deleted from nt position 2359 to 2796 with five bases added; pIJ6134 runs from nt position 2021 to 4346; and pIJ6140 runs from nt position 1 to 3430.

The work on which the present invention is based will now be described, by way of example only, with reference to these figures.

EXAMPLE 1 *scbA* - an *S. coelicolor* homologue of *afsA*

Alignment of the amino acid sequences of AfsA from *S. griseus* and its homologue, BarX, from *S. virginiae* (Kinoshita *et al.*, 1997), revealed two highly conserved regions (corresponding to amino acid residues 217-223 and 277-285 of AfsA). These sequences were used, with codon usage data derived from 64 Streptomyces genes (Wright and Bibb, 1992), to design degenerate oligonucleotides for use as primers in PCR. *Bam*HI sites were incorporated at the 5' end of each primer to allow subsequent cloning of the PCR product. An amplified fragment of the expected size (189 bp including the flanking BamHI sites) was obtained using *S. coelicolor* M145 DNA as template. The PCR product was cleaved with *Bam*HI, and cloned in the *Bam*HI site of the pUC19 derivative pIJ2925, yielding pIJ6114. Sequencing using universal and reverse primers revealed an *afsA* homologue of *S. coelicolor*, which was designated *scbA*.

The *Bam*HI insert of pIJ6110 was isolated and labelled with ^{32}P by random oligonucleotide priming and used as a hybridisation probe to isolate four cosmids from an

unaligned cosmid library of *S. coelicolor* M145 DNA. The probe failed to hybridise to the ordered cosmid library of Redenbach *et al.*, 1996 (see below). Digestion of the four cosmids with *Bam*HI revealed several restriction fragments of identical mobility, suggesting that each cosmid respresented the same genetic locus. Southern analysis of each of the cosmids using the same probe identified a common 4.5 kb *Bam*HI fragment and a smaller hybridising fragment that ranged in size from 2.5 kb to 3.0 kb. The 4.5 kb fragment and the 3.0 kb *Bam*HI fragment from cosmid GB10 were cloned in the *Bam*HI site of pIJ2925 to yield pIJ6111 and pIJ6114, respectively. The restriction map of the contiguous 7.5 kb region is shown in Fig.1. All four hybridising cosmids from the unaligned library were used as probes to identify their position in the combined physical and genetic map of the *S. coelicolor* chromosome. *scbA* was localised to the gap that lies at approximately 5 o'clock in the ordered cosmid library, in *Ase*I fragment B, and next to cosmid 2H4 (H.M.Kieser personal communication; Kieser *et al.*, 1992).

EXAMPLE 2: *scbA* lies adjacent to genes likely to be involved in γ-butyrolactone synthesis and binding

The nucleotide sequence of the 7.5 kb *scbA* region was determined (the sequence has been deposited under EMBL accession number AJ007731). Frame analysis (Bibb *et al.*, 1983) revealed open reading frames (ORFs) with predicted translation products that showed homology to proteins likely (by analogy to the *S. griseus* system above) to be involved in both γ-butyrolactone synthesis and perception. ScbA (corresponding to nucleotide positions 2914-1970 of EMBL AJ007731 and 2142-1199 of Fig. 14) shares 64% amino acid sequence identity with AfsA, and

about 40% identity with other AfsA homologues. The deduced amino acid sequence of ScbR, a protein encoded by a divergent ORF (corresponding to nucleotides 3032-3679 of EMBL AJ007731 and 2261-2908 of Fig. 14) which is adjacent to *scbA*, shows high levels of similarity to several γ-butyrolactone binding proteins. It is 56% identical to FarA of *S. lavendulae* FRI-5, and 45% identical to ArpA. Each of these homologues possesses an N-terminal DNA-binding domain that is also found in the TetR family of transcriptional repressors. The C-terminal regions of the ScbR family of proteins are relatively poorly conserved, and the inventors propose that this may reflect their ability to bind different γ-butyrolactones.

Downstream of ScbR, and transcribed in the opposite orientation, lies ScbB (nucleotides 3795-4529 of EMBL AJ007731 and 3024-3758 of Fig. 14), whose predicted product shows 50% amino acid identity to a C-5 ketoreductase from *S. avermitilis* (Ikeda *et al.*, 1999); based on BLAST search (Altschul *et al.*, 1997).

EXAMPLE 3: Transcription of *scbA* and *scbR* occurs in a growth-phase-dependent manner

S1 nuclease protection experiments were carried out to determine the transcriptional start sites of *scbA* and *scbR* using RNA isolated from *S. coelicolor* M145 cultured in SMM to different stages of growth. A 259 bp PCR product (nucleotides 2786-3055 of EMBL, 2015-2284 of Fig. 14) labelled uniquely at the 5' end at position 2786/2015 was used as a probe for *scbA* transcripts, while a 280 bp PCR product (nucleotides 2894-3174 of EMBL, 2123-2403 of Fig. 14) labelled uniquely at the 5' end at position 3174/2403, was used as a probe for *scbR*. Putative

transcriptional start sites were identified 46 nucleotides upstream of the likely translational start site of *scbA*, and 123-124 nucleotides upstream of that for *scbR* (Fig 2a), i.e. at nucleotides 2960 and 2909-8, respectively (of the EMBL sequence, 2189 and 2138-7 of Fig. 14). Transcription of *scbA*, which was undetectable during exponential growth, increased markedly at late transition phase, and fell quickly as the culture entered stationary phase (Fig. 2b). The *scbR* transcript, while detectable during exponential growth, increased markedly in level in late transition phase, approximately one hour after the increase in the level of the *scbA* transcript. It also fell in stationary phase, after the decline in the level of the *scbA* transcript. The transcript of the major and essential sigma factor gene, *hrdB*, was used as a control, and was present at essentially constant levels through exponential growth, and fell gradually upon entry into stationary phase.

EXAMPLE 4: Binding of ScbR to the promoter regions of *scbA* and *scbR* is prevented by SCB1

scbR was expressed in *E. coli* JM101 by cloning the 1.2kb *Hinc*II-*Pvu*II fragment containing *scbR* (Fig. 1) in pIJ2925, yielding pIJ6120. Extracts of JM101/pIJ6120 were then used in gel retardation assays with a 5' end-labelled PCR product that contained the *scbA* and *scbR* promoter regions (Fig.3a). Retardation of the *scbAR* promoter fragment was readily detected on addition of the JM101/pIJ6120 extract (indicating binding of ScbR to the promoter region); no retardation was observed if the extract was first boiled, or with extract isolated from JM101 (Fig.3b). Addition of an excess of unlabelled PCR product resulted in a reduction in the proportion of the labelled promoter-containing fragment that was retarded;

however, no competition was apparent when unlabelled *Streptomyces* DNA (the plasmid pIJ922) was added, indicating a specific interaction between ScbR and the promoter DNA.

SCB1 (which, like A-factor, is a GBL) and its three chemically synthesized stereoisomers (Takano *et al.*, 2000) were added to the gel retardation assays to assess their ability to influence the DNA-binding activity of ScbR. Formation of the DNA-protein complex was markedly reduced by addition of 1µg of SCB1, while addition of equivalent amounts of each of the stereoisomers had little or no effect (Fig 3c). Equivalent amounts of A-factor, IM-2 and VB also failed to inhibit the DNA-binding activity of ScbR (data not shown) indicating that the specificity of ScbR for SCB1, its cognate γ-butyrolactone, is high.

DNase I footprinting was used to determine the location of the DNA sites to which ScbR binds. Two protected regions were identified (Fig 4a and b); one lies at nucleotide position -4 to -33 with respect to the *scbA* transcriptional start site (i.e. nucleotides 2964-2993 of the EMBL sequence, 2193-2222 of Fig. 14), while the other lies at nucleotide position -41 to -67 with respect to the *scbR* transcriptional start site (i.e. nucleotides 2867/8-2841/2 of EMBL, 2096/7-2070/1 of Fig. 14). Dilution of the JM101/pIJ6120 extract suggests that ScbR has a stronger affinity for the binding site upstream of *scbA* than for that upstream of *scbR*.

EXAMPLE 5: Deletion of *scbA* abolishes γ-butyrolactone synthesis, but results in overproduction of Act and Red, while deletion of *scbR* also abolishes γ-butyrolactone synthesis, but causes delayed Red

production

To assess the role of *scbA* and *scbR* in antibiotic production in *S. coelicolor*, in-frame deletions were made in each gene. Mutant *scbA* and *scbR* alleles were constructed in which most of the *scbA* and *scbR* coding regions (amino acids 42-276 out of 315, and 33-178 out of 216, respectively) were deleted . The mutant *scbA* and *scbR* alleles were cloned in the *E. coli* plasmid pKC1132, yielding pIJ6140 and pIJ 6134, respectively (Fig. 1), and introduced into *S. coelicolor* strain M145 by conjugation; selection for apramycin resistance ensured integration of the non-replicating plasmids into the streptomycete chromosome by homologous recombination. After three rounds of sporulation on non-selective medium, apramycin-sensitive segregants were screened by PCR, and putative *scbA* (M751) and *scbR* (M752) deletion mutants further confirmed by Southern analysis.

Confluent lawns of the parental strain M145, M751 (Δ*scbA*) and M752 (Δ*scbR*) were grown on nitrogen-limited SMMS agar plates to assess the affect of each deletion (Fig. 5). After 20 h, Red production had just begun in M145, while Act synthesis was undetectable. In contrast, M751 had produced large amounts of both Red and Act (detectable by exposing the agar plate to ammonia fumes, which resulted in the blue pigmentation characteristic of Act), and M752 had failed to produce either antibiotic. By 40 h, the overproduction of both Act and Red by M751 was very marked, while Red production was noticeably delayed and Act production detected earlier (Fig. 5A) in M752 as compared to M145. The mutant phenotypes were also observed on rich R5 agar and on phosphate-limited R2 agar, but both mutants resembled the parental strain on rich SFM agar and minimal medium containing mannitol as

carbon source. Growth of the strains in SMM liquid medium gave phenotypes that corresponded to those observed with SMMS agar.

To assess the ability of the mutants to respond to SCB1, 1 µg of chemically synthesised SCB1(Takano et al (2000) supra) was spotted onto confluent lawns of M751, M752 and M145 (Fig. 5b). While M145 responded in the expected way to exogenous SCB1, M752 did not respond. Since M751 precociously overproduced both Act and Red, it was not possible to determine whether it had retained the ability to respond to the the γ-butyrolactone; however, the inhibitory effect of high concentrations of SCB1 on antibiotic production in M145 (the lighter halo surrounding the point of application; Takano *et al.*, 2000) was also observed with M751, suggesting that it had indeed retained the ability to sense SCB1.

The ability of the mutants to produce compounds, including SCB1, with antibiotic stimulatory activity was assessed using the standard bioassay (the ability to induce precocious Act and Red production in a lawn of M145). M751, M752 and M145 were grown on SMMS agar and in SMM liquid medium, and samples of agar and culture supernatant from different growth phases were extracted with ethyl acetate. Neither mutants produced stimulatory activity (Fig. 6) regardless of growth phase or medium .

To confirm that the mutant phenotypes reflected the absence of a functional *scbA* or *scbR*, rather than a mutation elsewhere in the genome, *scbA* or *scbR* were reintroduced into M751 and M752, respectively. A 1194 bp PCR product containing *scbA* and its promoter (Fig. 1, pIJ6143), and a 1.3 kb *Bgl*II fragment containing *scbR* and its promoter (Fig.1, pIJ6135), were cloned in *E. coli* in

pSET152 yielding pIJ6147 and pIJ6135, respectively. The plasmids were introduced into the corresponding *S. coelicolor* mutant by conjugation and selection for apramycin resistance, and integration at the ΦC31 attachment site was confirmed by Southern hybridisation. All of the mutant phenotypes were restored to those observed in M145 (Fig. 6 for restoration of SCB1 synthesis).

EXAMPLE 6: *scbR* regulates the transcription of both *scbR* and *scbA*, and *scbA* is required for the transcription of *scbA*

Since ScbR binds *in vitro* to the *scbA* and *scbR* promoter regions, and given that SCB1 is able to prevent such binding, the effect of the *scbA* and *scbR* deletions on transcription of each of the genes *in vivo* was assessed. RNA was isolated from SMM-grown M145, M751 and M752 cultures at different stages of growth and subjected to S1 nuclease protection experiments (Fig. 7a). While the *scbA* transcript was readily detected in early transition phase cultures of M145, it was absent in M751(Δ*scbA*) and barely detectable in M752 (Δ*scbR*) regardless of growth phase, suggesting that both *scbA* and *scbR* are required for induction of *scbA* transcription. While the level of the *scbR* transcript increased during late transition and early stationary phase in M145, it was markedly diminished in the *scbA* mutant, and highly abundant in exponential and early transition phase in the *scbR* mutant. These observations suggest that *scbR* negatively regulates its own transcription, and that relief of this repression requires *scbA*. Transcription of *hrdB*, the major and essential sigma factor of *S. coeliclor*, was monitored as a control. Antibiotic production (Fig. 7a) and production of antibiotic stimulatory factors (Fig.

7b) were also assessed at the times of RNA extraction. The commencement of factor synthesis in M145 corresponded well with the increase in the *scbA* transcription.

EXAMPLE 7: Addition of SCB1 to M751 (Δ*scbA*) stimulates *scbR* transcription but fails to restore *scbA* transcription

To assess the effect of addition of exogenous SCB1 on *scbA* and *scbR* expression in the *ΔscbA* mutant, in which transcription of both genes is markedly impaired, chemically synthesised SCB1 was added at a final concentration of 31ngml^{-1} to a mid-exponential phase (OD_{450nm}= 0.5) culture of M751. While there was a marked increase in the level of *scbR* transcription, *scbA* transcription in the *ΔscbA* mutant was not restored (Fig. 8).

EXAMPLE 8: Deletion of *scbA* in *S. lividans* 1326 abolishes gamma-butyrolactone synthesis and results in increased production of Act and Red by strains containing the multi-copy plasmids pIJ68 or pIJ6014.

The mutant *scbA* allelle from *S. coelicolor* (described in example 5) was introduced into *S. lividans* 1326 using pIJ6140. Integration of the non-replicating plasmid was selected using apramycin. After three rounds of non-selective growth (on SFM agar) colonies were screened for sensitivity to apramycin (indicating loss of the plasmid due to a second homologous recombination event). 4 apramycin-sensitive colonies were identified among 3,000 colonies screened. PCR analysis of chromosomal DNA produced amplified DNA fragments consistent with that observed from wild type chromosomal DNA for three colonies, whereas the fourth colony yielded a smaller DNA

fragment consistent with the in-frame deletion allelle. Southern hybridisation experiments of chromosomal DNA digested either with *NcoI* or a mixture of *BglII* and *PstI* produced hybridising bands consistent with the results expected for the wild type arrangement for the first three colonies and the mutant for the fourth colony, which was designated *S. lividans* M707. When this strain was grown on agar medium no ScbA was detected, whereas it was demonstrably produced by the wild type *S. lividans* 1326 strain.

Spores of the M707 strain were inoculated into liquid YEME medium (containing 0.5% glycine and 5mM $MgCl_2$) and grown with shaking at 30°C for 2 days. The mycelium was collected by centrifugation and used to produce protoplasts, which were transformed with pIJ68 (actII-orf4) (Passantino R et al (1991) J. Gen Microbiol 137:2059 - 2064), pIJ6014 (redD) (Takano E et al (1992) 6(19): 2797 - 2804) or pIJ486 (vector control) (Ward J M et al (1986) Mol Gen Genet 203:468 - 478). Thiostrepton-resistant transformants were selected and tested in shake flask fermentation experiments. Spores of the transformed strains were streaked on SFM agar (containing 50 µg/ml of thiostrepton) and incubated at 30°C for 4-5 days. Spores were harvested and inoculated into spring flasks with 50ml YEME (containing 50 µg/ml of thiostrepton). After two days incubation at 30°C the mycelium was collected by centrifugation and resuspended in fresh spring flasks containing phosphate-limited Evans medium with 20 µg/ml of thiostrepton. Incubation was continued at 30°C for a further seven days with 1ml samples being removed for assessment of antibiotic production. The Act or Red production is shown in Figures 12 and 13 and confirmed the findings observed for *S. coelicolor* that antibiotic synthesis was precocious and elevated. Approximately

three to four times the concentration of Red was observed compared to the *S. lividans* 1326 strain carrying pIJ6014. For Act the concentration difference was five to ten fold for the pIJ68-containing strains. Moreover, when dry cell weight (DCW) measurements were made it was noted that the M707/pIJ68 strain produced less mycelial material than the *S. lividans* 1326/pIJ68 under these conditions. Thus, when expressed as concentration of Act produced per gram DCW, the M707 strain produced 121 compared to the control 1.5.

(Plasmids pIJ68 / pIJ6014 were introduced into *S. lividans* to supply the pathway specific transcriptional activator genes for Act / Red production. No equivalent plasmids are required in *S. coelicolor*.)

These strains were further tested for their ability to produce Act in 1 litre liquid batch fermentations in stirred tank bioreactors using a modified phosphate-limited Evans medium (with NH_4Cl instead of $NaNO_3$). The M707/pIJ68 strain produced 10g/l of act compared to the *S. lividans* 1326/pIJ68 control, which made 5g/l.

DISCUSSION

Two genes, *scbA* and *scbR*, have been isolated from *S. coelicolor* A3(2) and respectively show high homology to the *afsA* and *arpA* genes of *S. griseus*, which encode A-factor synthetase and A-factor binding protein. The in-frame deletion mutant of *scbA* overproduces both antibiotics, while the in-frame deletion mutant of *scbR* is delayed in RED production and does not produce γ-butyrolactones which (in the wild-type strain) cause precocious RED and ACT production. These phenotypes are most surprising considering the high homology of the

genes to the A-factor system in *S. griseus*.

Onishi *et al.* (1999) reported the cascade for the streptomycin production in *S. griseus*, triggered by A-factor. ArpA (A-factor binding protein) binds to the promoter region of *adpA* (a transcriptional activator for streptomycin production) and represses the transcription of *adpA* during early growth cultures. In transition phase, A-factor is synthesised via AfsA and releases the ArpA from the promoter region by binding to it. Thus *adpA* is transcribed and activates the streptomycin biosynthetic cluster via *strR* (streptomycin pathway-specific activator) and the antibiotic is produced. To corroborate their model, the *afsA* mutant (equivalent to the *scbA* mutant of the present work) produces neither streptomycin nor A-factor. Also the *arpA* mutant (equivalent to the *scbR* mutant of the present work) overproduces antibiotics; A-factor production is not effected. These are the reverse phenotypes compared to those of the in-frame deletion mutants of the present work using *S. coelicolor*. The inventors propose that γ-butyrolactones are involved in antibiotic production differently in *S. coelicolor*, compared with the known GBL model of *S. griseus*.

The two genes *scbA* and *scbR* are located next to each other in the *S. coelicolor* genome, which is not the case for the equivalent genes of *S. griseus* (*afsA* and *arpA*). *afsA* is located at the end of the linear chromosome (Lezhava *et al.*, 1997) thus being easy to mutate to obtain deletion mutants and *arpA* is located elsewhere on the chromosome (Ohnishi *et al.*, 1999). On the other hand, like the arrangement in *S. coelicolor*, the homologues of *afsA* in *S. virginiae* (*barX*) and *S.fradiae* (*farX*) are located next to genes encoding γ-butyrolactone

binding proteins (*barA* and *farA* respectively) (Nakano *et al.*, 1998; Waki *et al.*, 1997). In *S. virginiae*, a mutation in *barA* (a homologue of *arpA*) results in precocious virginiamycin production, consistent with the role of *arpA* in *S. griseus*, yet it abolishes VB production (Nakano *et al.*, 1998). The inventors propose, therefore, that the juxtaposition of streptomycete genes encoding GBL synthetases and GBL binding proteins may be reflective of a different antibiotic regulatory system from that of the *S. griseus* model (possibly in some cases additional to such a regulatory system), namely one in which functional deletion of the gene encoding the GBL binding protein leads not to overproduction of antibiotic (as in the *S. griseus* system), but under- or delayed production. Moreover, they suggest that in such systems, functional deletion of the GBL synthetase leads to overproduction of the antibiotic (in contrast to abolition of streptomycin production in *S. griseus*).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

S coelicolor A3(2) strain M145 (Hopwood *et al.*,1985), M751 and M752 (this study) were manipulated as previously described (Hopwood *et al.*, 1985). *E.coli* K-12 strains JM101(Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were grown and transformed according to Sambrook *et al.*, (1989). Vectors used were pIJ2925 (Janssen and Bibb., 1993), pKC1132 (Bierman *et al.*, 1992), pset152 (Bierman *et al*, 1992), pBluescript SK^+ (Stratagene), pGEM-T vector (Promega). SMM is the modified minimal medium of Takano *et al.*, (1992); it lacks $(NH_4)_2SO_4$ and has 0.25 mM NaH_2PO_4, 0.25mM K_2HPO4 instead of 0.5mM each. SMMS is a modified solid version

of SMM, as described above. SFM medium was used to make spore suspensions and for use in conjugation with *E.coli* ET12567 containing the RP4 derivative pUZ8002 (Flett *et al.*, 1997).

PCR

The synthetic oligonucleotides oligo1; 5'-GACCACGT(CG)CC(CG)GGCATG and oligo2; 5'-GTCCTG(CG)TGGCC(CG)GT(CG)AC(CG)CG(CG)AC (bracketed nt indicate positions of degeneracy) were used in the PCR (Erlich, 1989) to amplify the internal segment of *scbA* from *S. coelicolor* M145 total DNA (Fig. 2a). The reaction mixture contains: 10x reaction mixture supplied by Boehringer Mannheim, 200µM final concentration of four dNTPs, 5% final concentration of DMSO, 50pmol of each primer, 50ng of chromosomal DNA in a final volume of 100µl. After denaturation by boiling 5 min, 2.5U of Taq polymerase was added and subjected to 30 cycles of denaturation at 94°C for 50 sec, annealing at 55°C for 40 sec and extension at 72°C for 40 sec, and then incubated at 72°C for 10 min. PCR products were analyzed on a 2% w/v agarose gel electrophoresis.

To complement M751, *scbA* coding sequence with its promoter region was amplified by PCR from *S. coelicolor* M145 cosmid GB10 DNA. Two synthetic oligonucleotides 5'-GCCAGCAGGTGGGCGACCTGAC (1796nt position) and 5'-GATCGCCCGGTCCTGCTTGGCCATG (3055nt position) were used. The PCR conditions were as stated above except the High Fidelity Kit (Beoringher Mannheim) was used and the PCR cycle was reduced to 20. The PCR product was purified by a Sephadex G-50 (Pharmacia) spin column then ligated to the pGEM easy vector (Promega) and transformed to JM101.The sequence of the transformant was confirmed by

using the ABI automated sequencer and Big Dye dye terminator cycle sequencing kit (Perkin Elmer).

Nucleotide sequencing

The nucleotide sequencing of the 7.5kb *scbA* region was sequenced by the ABI automated sequencer and using the Big Dye dye terminator cycle sequencing kit (Perkin Elmer) as recommended by the suppliers, except in the PCR reaction, final concentration of 5% DMSO was added to the reaction mixture. The sequence was submitted to the databases (EMBL AJ007731) and sequenced on both strands.

S1 nuclease mapping

For each S1 nuclease reaction, 30 or 40µg of RNA were hybridized in NaTCA buffer (Murray, 1986; Solid NaTCA(Aldrich) was dissolved to 3M in 50mM PIPES, 5mM EDTA, pH7.0) to about 0.002pmol (approximately 10^4Cerenkov counts min 10^{-1}) of the following probes. For scbA the synthetic oligonulceotide 5'-TATCCAGCTGACCGGGAACGCGTC, corresponding to the region within the coding region of *scbA* was labelled with [^{32}P]-ATP using T4 polynucleotide kinase uniquely at the 5' end of the oligonucleotide, then used in the PCR reaction with the unlabelled oligonucleotide 5'-ATCGCCCGGTCCTGCTTGGCCATG which corresponds to a region upstream of the *scbA* promoter region to generate a 259bp probe. For *scbR*, the synthetic oligonulceotide 5'-AAGTAGAGGGCTCCCTTGGTCA, corresponding to the region within the coding region of *scbR* was labelled with [^{32}P]-ATP using T4 polynucleotide kinase uniquely at the 5' end of the oligonucleotide, then used in the PCR reaction with the unlabelled oligonucleotide 5'-CAAAACTACTGCTTCGGGCATG which corresponds to a region upstream of the *scbR* promoter

region to generate a 280bp probe. Both PCR reactions were done using M145 total DNA as template. For *hrdB*, the probe was made as previously described (Buttner *et al.*, 1990). Subsequent steps were as described by Strauch *et al.* (1991).

Gel retardation assays and Dnase I footprinting studies

50pmol of the synthetic oligonucleotides 5'-CTGCACCCTGGTCCGGTGGACA and 5'-ATCGCCCGGTCCTGCTTGGCCATG were both labelled with [^{32}P]-ATP using T4 polynucleotide kinase uniquely at the 5' end of the oligonucleotide, then used in the PCR reaction with the unlabelled synthetic oligonucleotide corresponding to the other primer to generate a 244bp DNA fragment. The PCR amplified fragment was further purified by Qiagen PCR purification kit. The gel retardation assay reaction mixture contains; 5x gelretardation buffer(125mM HEPES pH7.5, 20mM DTT, 10mM ATP, 20% glycerol) 200mM KCl, 0.16µg/µl calf thymus DNA, and 0 to 15µl of JM101 crude extract containing ScbR protein in a final volume of 12.5 to 25 µl. The final concentration of DNA fragments used was 2.5 ng/ml. The mixture was incubated at room temperature for 10 min then 2 µl of dye(50%(w/v) glycerol with BPB in TE) was added to the mixture and 10µl was loaded to a 5%(w/v) non-denaturing polyacrylamide gel buffered with TBE. SCB1 was added to the reaction mixture either prior to incubation, or after 10 min of incubation then incubated for further 10 min.

Dnase I footprinting studies were performed as described by Drapal and Sawer, (1995). 25 ng/ml of DNA fragments were incubated in gel retardation assay mixture (final total volume 25µl) with varying concentration of protein. After incubation, 25µl of 10mMMgCl and 5mM$CaCl_2$ was added. After 1 min 0.1unit of Dnase I (Boerhinger Mannheim) was added and incubated for 45 sec then the reaction terminated by adding 30µl of stop solution (20mMEDTA, 200mM NaCl, 1% SDS(w/v), 250µg ml^{-1} tRNA). The DNA fragments were purified by phenol/chloroform extraction and precipitated with three volumes of ethanol. The precipitants were resuspended in loading buffer and ran on a 6%(w/v) sequencing gel. Sequencing reactions were

performed using the synthesised oligonucleotides as primers on double strand DNA and by using a dideoxy sequencing kit (Taq Track, Progema).

Crude extract isolation

An overnight culture of *E. coli* JM101 harboring pIJ6120 was diluted 1/100 and innoculated into 25ml LB media. The culture was grown at 37°C for approximately 3.5 hr or until the cultures were at 1.0 OD_{600nm}. The culture was then induced with final concentration of 1mM IPTG. After further 3 hr of growth, the cells were harvested by centrifugation and the cell pellet was washed twice with buffer (50mM Tris pH7.0, 1mM EDTA, 1mM DTT, 100mM PMSF), resuspended in 500µl of buffer and disrupted by sonication. The cell lysate was then clarified by centrifugation and the supernatant was used as crude extracts.

Isolation of γ-butyrolactones, bioassay and HPLC analysis

γ-butyrolactones were isolated from liquid or solid media by extracting the culture supernant or the agar with ethylacetate. The ethylacetate was evaporated and the sample was resuspended in 100% methanol for use in a bioassay or for HPLC analysis. Bioassay and HPLC analysis were conducted as described previously(Takano *et al.*, 2000).

Construction of an in-frame deletion mutant of scbA *and* scbR

The in-frame deletion mutant of *scbA* was constructed by digesting pIJ6136 which contains a 1.4kb flanking DNA of *scbA* in pIJ2925 (Fig. 1) with *Bam*HI and end filled using Klenow fragment and ligated with a 1.1kb *Pvu*II -*Hinc*II fragment from pIJ6111. The transformants were analysed to find the *Pvu*II -*Hinc*II fragment was inserted with the internal *Pst*I site at the *EcoR*I side of the multiple cloning site of pIJ6136 and designated pIJ6137. The *Bgl*II fragment of pIJ6137 was inserted into the *Bam*HI site of pKC 1132 (Bierman *et al.*, 1992) to give pIJ6140 (Fig. 1). The in-frame deletion mutant of *scbR* was constructed by PCR using the High Fidelity Kit (Beoringher Mannheim) with a universal primer and 5'-CATCTGCAGCGTGATCGTGGCAGCTTGGTAG (3130nt position) primer designed to give a 1.059kb DNA fragment flanking *scbA* as described earlier. A *Pst*I site was designed into the end of this fragment to enble ligation with a *Pst*I site internal of *scbR*. pIJ6111 was used as template for the PCR reaction and the amplified product was cloned into pGEM-T vector (Promega) to give pIJ6148. The sequence of the PCR amplified insert of pIJ6148 was confirmed by ABI automated sequencing. The *Bam*HI-*Kpn*I 3kb fragment of pIJ6111 was cloned into pBluescript SK^+ (Stratagene) to give pIJ6131. The 1.059kb *Bam*HI-*Pst*I fragment was isolated from pIJ6148 and cloned into the *Bam*HI-*Pst*I digested pIJ6131 to give pIJ6152. pIJ6152 was then digested with *Kpn*I and blunt ended then further digested with *Bam*HI. This 2.48kb DNA fragment was cloned into pKC1132 digested with *Bam*HI and *Eco*RV to give pIJ6134 (Fig. 1). Both plasmids were introduced into the methylation deficient *E. coli* strain ET 12567 containing the RP4 derivative pUZ8002 (Paget *et al.*, 1999) and transferred into *S. coelicolor* M145 by conjugation. Single-crossover exconjugants were selected on SFM containing apramycin. Three such single colonies were

then taken through three rounds of non-selective growth on SFM to promote the second crossover. Spores were then plated for single colonies which were scored for apramycin sensitivity. Deletions within *scbA* and *scbR* were confirmed by PCR using primers correponding to flanking sequences, and by Southern hybridisation. For *scbA*, nine out of 20 apramycin sensitive colonies were deleted for *scbA* while 11 had reverted to wildtype. For *scbR*, 4 out of 20 apramycin sensitive colonies were deleted for *scbR* while 16 reverted to wildtype. The *scbA* and *scbR* deletion mutants were called M751 and M752, respectively.

To complement the mutants, a 1194bp PCR product (subsequently sequenced) containing the entire *scbA* coding region with its promoter (pIJ6143) and a 1.3kb *Bgl*II fragment containing the entire region of *scbR* with its promoter (pIJ6135) (Fig. 1) was cloned into a conjugative vector pset152 (Bierman *et al*, 1992), which integrates into the chromosome of *S. coelicolor* by site-specific recombination at the bacteriophage ΦC31 attachment site, *attB* (Kuhstoss, E. *et al* 1991). The resulting plasmids, pIJ 6147 and pIJ6135 (Fig. 1), respectively were transferred into *S. coelicolor* by conjugation via the *E. coli* donor ET 12567 containing the RP4 derivative pUZ8002 (Paget *et al.*, 1999). Exconjugants were purified by single-colony isolation, and the plasmid integration were confirmed by southern hybridization.

Other methods

Antibiotic production was determined by extracting actinorhodin and undecylprodigiosin as described previously (Strauch *et al.*, 1991). RNA was isolated as described in Strauch *et al.*, (1991). Southern

hybridisation was done as previously described (Hopwood *et al.*, 1985). Probes for southern hybridisation were made by labelling DNA fragments or PCR products with ^{32}P by random oligolabelling (Pharmacia).

Further protocols are performed according to standard reference texts, such as Hopwood et al. (1985) and Sambrook et al. (1989), or later editions thereof.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. In particular, although the claims refer to certain species of *Streptomyces*, it will be readily apparent that the teaching of the invention may be applied to other species of *Streptomyces*, especially species which are closely related to the species referred to in the claims and/or species having a similar arrangement of *scbA* and *scbR* genes (or their homologues).

REFERENCES

Altschul, S. F., et al. (1997) *Nucleic Acids Res.* **25:** 3389-3402

Ando, N., et al. (1997) *J. Antibiot* **50**: 847-852.

Bate, N., et al. (1999) *Chemistry & Biology* **6:** 617-624.

Bibb, M.J., et al. (1984) *Gene* **30:** 157-166.

Bierman, M., et al. (1992) *Gene* **116:** 43-49.

Buttner, M.J., et al. (1990) *J Bacteriol* **172:**3367-3378.

Chater, K.F. and Bibb, M.J. (1997) Regulation of bacterial antibiotic production. In *Biotechnology, volume 7: Products of Secondary Metabolism.* Kleinkauf, H. and von Döhren, H. (eds). Weinheim, VCH, Germany. pp 57-105.

Chater K.F., and Hopwood D.A. (1993) *Streptomyces*. In Bacillus subtilis *and other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular genetics.* Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds.). Washington, D.C.: American Society for Microbiology, pp. 83-89.

Chakraburtty, R., et al. (1996) *Mol Microbiol* **19:**357-368.

Chakraburtty, R. and Bibb, M. (1997) *J Bacteriol* **179:** 5854-5861.

Drapal, N. and Gary, S. (1995) *Mol Microbiol* **16:**597-607.

Erlich, H.A. (1989) *PCR Technology.* New York: Stockton Press.

Fouces, R. et al. (1999) *Microbiol* **145:** 855-868.

Flett, F., et al. (1997) *FEMS Microbiol Lett* **155:** 223-229

Gramajo, H.C., et al. (1993) *Mol Microbiol* **7:**837-845.

Hara, O., et al. (1983) *J Gen Microbiol* **129:**2939-2944.

Hopwood, D.A., et al. (1985) *Genetic Manipulation of* Streptomyces: *A Laboratory Manual*. Norwich: John Innes Foundation.

Hopwood, D.A., et al. (1995) Genetics of antibiotic production in *Streptomyces coelicolor* A3(2). In: *Genetics and Biochemistry of Antibiotic Production*. Vining, L. (ed) Butterworth-Heinemann, Newton, MA, USA. pp. 65-102.

Horinouchi, S., and Beppu, T. (1994) Autoregulators. In: *Genetics and Biochemistry of Antibiotic Production*. Vining, L. (ed) Butterworth-Heinemann, Newton, MA, USA. pp. 103-119.
Horinouchi, S., et al. (1985) *J Antibiot* **36:**636-641.
Horinouchi, S., et al. (1989) *J Bacteriol* **171:**1206-1210.
Ikeda,H., et al. (1999) *Proc. Natl. Acad. Sci.* **17**: 9509-9514
Janssen, G.R., and Bibb,M.J. (1993) *Gene* **124:** 133-134.
Kieser, H.M., et al. (1992) *J Bacteriol* **174:**5496-5507.
Kieser, H.M., et al. (eds) (2000) Practical Streptomyces Genetics, John Innes Foundation, Crowes Printers, Norwich, England.
Kinoshita, H., et al. (1997) *J Bacteriol* **179**: 6986-93.
Kitani, S., et al. (1999) *J Bacteriol* **181:** 5081-5084.
Kondo, K., et al. (1989) *J. Antibiot* **42**: 769-778.
Kuhstoss, S., and Rao, R.N. (1991) *J Mol Biol* **222:** 897-908.
Lezhava, A., et al. (1997) *Mol Gen Genet* **253**: 478-483.
MacNeil, D.J., et al. (1992) *Gene* **155:** 119-125.
Miyake, K., et al. (1990) *J. Bacteriol* **172**: 3003-3008.
Mori, K. (1983) *Tetrahedron Lett* **39:**3107-3109.
Murray, et al. *Anal Biochem* **158(1)**: 165-70.
Nakano, H., et al. (1998) *J Bacteriol* **180**: 3317-3322.
Nihira, T., et al. (1988) *J Antibiot* **41:**1828-1837.
Okamoto, S., et al. (1995) *J. Biol Chem* **270**: 12319-12326.
Onaka, H., et al. (1995) *J Bacteriol* **177:**6083-6092.
Onaka, H., and Horinouchi, S. (1997) *Mol Microbiol* **24:**991-1000.
Onaka, H., et al. (1998) *Mol Microbiol* **28:** 743-753.
Ohnishi, Y., et al. (1999) *Mol Microbiol* **34:**102-111.
Paget, M.S.B, et al. (1999) *J Bacteriol* **181:**204-211.
Redenbach, et al. (1996) *Mol Bicrobiol* **21**: 77-95.
Rothstein, et al. (1987) *Gene* **53(2-3)**: 153-61.
Ruengjitchatchawalya, M., et al. (1995) *J Bacteriol* **177**: 551-557.

Sambrook, J., et al. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Sato, K., et al. (1989) *J Ferment Bioeng* **68:**170-173.
Strauch, E., et al. (1991) *Mol Microbiol* **5:** 289-298.
Takano, E., et al. (1992) *Mol Microbiol* **6:**2797-2804.
Takano, E., et al. (2000) J.Biol.Chem. 275:11010-11016.

Waki, M., et al. (1997) *J Bacteriol* **16**:5131-5137
Wright, F., and Bibb, M.J. (1992) *Gene* **113**:55-65.
Yamada, Y. (1999) Auto regulatory factors and regulation of antibiotic production in *Streptomyces*. In *Microbial signalling and communication*. England, R., Hobbs, G., Bainton, N., and Roberts, D. McL. (eds.) Cambridge: the Society for General Microbiology, pp. 177-196.
Yamada, Y., et al. (1987) *J Antibiot* **40:**496-504.

All of the above references (and any later editions thereof) are hereby incorporated by reference in their entirety, individually and for all purposes.